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(54) Title: MICROBIAL 9α-HYDROXYLATION OF STEROIDS

(57) Abstract: A method is described to construct genetically modified strains of steroid degrading micro-organisms wherein multiple genes involved in steroid nucleus degradation are inactivated, such as steroid dehydrogenase genes. Examples for such genes are kstD1 and kstD2: strains with multiple inactivated steroid degrading enzyme genes can be used in the accumulation of steroid intermediates with a high yield. A preferred accumulation product is 9α-hydroxy-4-androstene-3,17-dione.

Microbial 9α-hydroxylation of steroids

The invention relates to a method to prepare genetically modified micro-organisms having inhibited capacity for nucleus degradation of steroids, the use of such micr-organism in steroid accumulation as well as such modified micro-organisms.

The ability to degrade phytosterols is widespread in nocardioform actinomycetes and requires a set of enzymes degrading the side-chain and the steroid nucleus structure.

The enzyme 3-ketosteroid Δ^1-dehydrogenase (KSTD) [4-ene-3-oxosteroid:(acceptor)-1-ene-oxidoreductase, EC 1.3.99.4] is involved in cleavage of ring B of the steroid nucleus by introducing a double bond at the C1-C2 position. More particularly, the enzyme is involved in the conversion of 4-androstene-3,17-dione in 1,4-androstadiene-3,17-dione and of 9α-hydroxy-4-androstene-3,17-dione in 9α-hydroxy-1,4-androstadiene-3,17-dione (see Figure 1). The enzyme has been identified in several bacteria: *Arthrobacter simplex* (Penasse and Peyre, 1968 Rhodococcus. Crit Rev Biotech **14**:29-73), *Pseudomonas* (Levy and Talalay, 1959 J Biol Chem **234**:2009-20013; 1959 J Biol Chem **234**:2014-2021), *Nocardia restrictus* (Sih and Bennet, 1962 Biochem Biophys Acta **56**:587-592), *Nocardia corallina* (Itagaki *et al.*, 1990 Biochim Biophys Acta **1038**:60-67), *Nocardia opaca* (Drobnič *et al.*, 1993 Biochim Biophys Res Comm **190**:509-515), *Mycobacterium fortuitum* (Wovcha *et al.*, 1979 Biochim Biophys Acta **574**:471-479) and *Rhodococcus erythropolis* IMET7030 (Kaufmann *et al.*, 1992 J Steroid Biochem Molec Biol **43**:297-301). KSTD of *N. opaca* has been characterized as a flavoprotein (Lestrovaja *et al.*, 1978 Z Allg Mikrobiol **18**:189-196). Only the KSTD encoding genes (*kst*D: 3-ketosteroid Δ^1 Dehydrogenase) of *A. simplex, Comamonas testosteroni* and *Rhodococcus rhodochrous* have been fully characterized (Plesiat *et al.*, 1991 J Bacteriol **173**:7219-7227; Molnár *et al.*, 1995 Mol Microbiol 15:895-905; Morii *et al.*, 1998 J. Biochem 124:1026-1032).

The exclusive inhibition of the steroid 1,2-dehydrogenase causes accumulation of 9α-hydroxy-4-androstene-3,17-dione, an excellent starting material for corticoid synthesis (Kieslich K., 1985 J Basic Microbiol **25**:461-474). 9α-Hydroxyandrogens are of industrial importance as anti-androgens, anti-estrogens and antifertility. The 9α-hydroxy group is easily dehydrated to the 9(11)-dehydro system and offers a starting structure for the production of 9α-halogen corticoids.

Rhodococcus species are well-known for their large catabolic potential (Warhurst and Fewson, 1994 Rhodococcus. Crit Rev Biotech **14**:29-73; Bell *et al.*, 1998 J Appl Microbiol **85**:195-210). Several *Rhodococcus* species are able to degrade natural phytosterols, which are inexpensive starting materials for the production of bioactive steroids (Kieslich K., 1986 Drug Res **36**: 888-892). *Rhodococcus* and *Mycobacterium* strains treated with mutagens and/or incubated with enzyme inhibitors convert sterols into 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione (Martin, 1977 Adv Appl Microbiol **22**:29-58).

Although cloning of *kst*D and expression of an inactive KSTD protein of *R. erythropolis* IMET7030 in *Escherichia coli* have been described (Wagner *et al.*, 1992 J Basic Microbiol **32**:65-71; 1992 J Basic Microbiol **32**:269-277) and a nucleotide sequence of *N. opaca* (Drobnič *et al.*, 1993 Biochem Biophys Res Comm **190**:509-515) (synonym *R. erythropolis* IMET7030) is available (DDBJ/EMBL/GenBank U59422), no molecular characterization of this gene has been reported. KSTD activity is essential for steroid nucleus degradation and *kst*D gene inactivation is needed to accumulate steroid intermediates. According to one aspect of the present invention the nucleotide sequence of the *kst*D gene of *R. erythropolis* has been provided. KSTD protein is encoded by nucleotides 820-2329 of SEQ ID NO:1.

Inactivation of genes is a powerful tool for analysis of gene function and for introduction of metabolic blocks. Gene disruption with a non-replicative vector carrying a selective marker is the commonly used method for gene inactivation. Construction of strains with desirable properties via metabolic pathway engineering approaches, however, may require the stepwise inactivation or replacement of several genes. This is only possible when a suitable strategy for introduction of unmarked gene deletions or gene replacements, allowing infinite rounds of metabolic engineering without being dependent on multiple markers, is available. According to another aspect of the present invention there is provided a stepwise inactivation of genes, preferably dehydrogenase genes, involved in steroid degradation. In particular the invention applies for an inactivation of genes involved in the accumulation of 9α-hydroxy-4-androstene-3,17-dione by growing of micro-organisms on 4-androstene-3,17-dione. Preferably, at least the gene *kst*D1 is inactivated.

It was unexpectedly found that disruption of the *kst*D1 gene encoding 3-ketosteroid Δ^1-dehydrogenase in *R.erythropolis* SQ1 did not result in inactivation of steroid nucleus degradation. The remaining activity appeared to be based on the presence of a second enzyme. It has now been found that inactivation of more than one gene is required to obtain a strain completely blocked in steroid nucleus degradation. Preferably the second

enzyme is a dehydrogenase, more preferably a KSTD isoenzyme. In order to make it possible to disrupt or delete several genes, preferably a method of site-directed mutagenesis can be used. A method for introduction of unmarked gene deletions is to be preferred for the stepwise inactivation of KSTD genes. The resulting genetically modified strains would be free of heterologous DNA.

According to another preferred embodiment of this invention, at least the gene *kst*D2 is inactivated. Most preferably, at least both the genes *kst*D1 and *kst*D2 are inactivated. Another aspect of the present invention is the nucleotide sequence of the *kst*D2 gene of *R. erythropolis*. KSTD2 protein is encoded by nucleotides 1-1678 of SEQ ID NO:5.

No methods for introduction of unmarked gene deletions in the genus *Rhodococcus* have been reported. Gene deletion or gene replacement methods, however, have been described for some other members of the actinomycetales, namely *Streptomyces* (Hillemann *et al.*, 1991 Nucleic Acid Res **19**:727-731; Hosted and Baltz, 1997 J. Bacteriol **179**:180-186), *Corynebacterium* (Schäfer *et al.*, 1994 Gene **145**:69-73) and *Mycobacterium* (Marklund *et al.*, 1995 J Bacteriol **177**:6100-6105; Norman *et al.*, 1995 Mol Microbiol **16**:755-760; Sander *et al.*, 1995 Mol Microbiol **16**:991-1000; Pelicic *et al.*, 1996 Mol Microbiol **20**:919-125; Knipfer *et al.*, 1997 Plasmid **37**:129-140). Counter-selectable markers may be used to screen for the rare second recombination event resulting in gene deletion or gene replacement. In this respect, both *sac*B and *rps*L proved to be useful reporter genes (Hosted and Baltz, 1997 J Bacteriol **179**:180-186; Schäfer *et al.*, 1994 J Bacteriol **172**:1663-1666; Sander *et al.*, 1995 Mol Microbiol **16**:991-1000; Pelicic *et al.*, 1996 Mol Microbiol **20**:919-925; Jäger *et al.*, 1992 J Bacteriol **174**:5462-5465), but other suitable markers can be used as well. The use of *rps*L in *Rhodococcus* has not been reported, but *sac*B (encoding the *Bacillus subtilis* levansucrase) provides a potent positive selection marker in this genus (Jäger *et al.*, 1995 FEMS Microbiol Lett **126**:1-6; Denis-Larose *et al.*, 1998 Appl Environ Microbiol **64**:4363-4367).

The *B. subtilis* levansucrase, encoded by the *sac*B gene, catalyzes hydrolysis of sugars and synthesis of levans (high-molecular weight fructose polymers). Expression of *sac*B in *Rhodococcus* is lethal in the presence of sucrose. The biochemical basis for toxicity of levansucrase action on sucrose is still unknown. Conditional lethality (i.e. presence or absence of sucrose) of the *sac*B gene therefore can be used as a counter-selectable marker. Counter-selection in this context means that expression of the marker is lethal, instead of giving rise to resistance as is the case for selectable markers (e.g. resistance markers).

Counter-selection is needed to select for those mutants that have undergone a second recombination event, thereby losing the *sac*B marker and introducing the desired mutation. The advantage of this system is that during selection solely potentially good mutants will survive the selection. Compared to a system in which only one selection marker is used, counter-selection avoids a time consuming screening process for loss of the resistance marker that would be necessary in an one-selection-marker system.

An advantage of unmarked mutation is that it allows the repetitive introduction of mutations in the same strain. Foreign DNA (vector DNA) is removed in the process of introducing the mutation. Newly introduced vector DNA, for the introduction of a second mutation, therefore cannot integrate at the site of the previous mutation (by homologous recombination between vector DNA's). Integration will definitely happen if vector DNA is still present in the chromosome and will give rise to a large number of false-positive integrants. The system enables the use of a sole antibiotic gene for the introduction of an infinite number of mutations. Unmarked mutation also allows easy use in the industry because of the absence of heterogeneous DNA allowing easy disposal of fermentation broth.

Gene inactivation by gene deletion enables the construction of stable, non-reverting mutants. Especially small genes (<500 bp) are inactivated more easily by gene deletion compared to gene disruption by a single recombination integration. Gene deletion mutagenesis can also be applied to inactivate a cluster of several genes from the genome. The gene deletion mutagenesis strategy can be applied also for gene-replacement (e.g. changing wild type into mutant gene).

The preferred strain for mutagenesis of the catabolic steroid dehydrogenases genes is *Rhodococcus erythropolis*. However, unmarked gene deletion of *kst*D1 and/or *kst*D2 in other species, genetically accessible by conjugation, is conceivable if the molecular organization is the same (or similar) as in *R. erythropolis* SQ1. Preferably these species belong to the genus *Rhodococcus* but also related species such as *Nocardia*, *Mycobacterium* and *Arthrobacter* can be used.

Gene inactivation in *Rhodococcus* is hampered by the occurrence of illegitimate recombination events resulting in random genomic integration of the mutagenic vector (Desomer *et al.*, 1991 Mol Microbiol 5:2115-2124; Barnes *et al.*, 1997 J Bacteriol 179:6145-6153), a phenomenon we encountered when attempting to disrupt the *kst*D1 gene in *R. erythropolis* SQ1. Illegitimate recombination is also a well-known phenomenon in some slow-growing species of *Mycobacterium* (McFadden, 1996 Mol Microbiol 121:205-211). Conjugative plasmid transfer from *E.coli* S17-1 to *Rhodococcus* has been shown to minimize random integration (Powell and Archer, 1998

Antinie van Leeuwenhoek 74:175-188). Conjugative mobilization of plasmids from *E. coli* strain S17-1 to many different strains of coryneform bacteria and to *Rhodococcus fascians* DSM20131 has been proven possible (Schäfer *et al.*, 1990 J. Bacteriol 172:1663-1666; Jäger *et al.*, 1995 FEMS Microbiol Lett 126:1-6). According to the present invention conjugative transfer of a mutagenic vector carrying the *sac*B gene as counter-selectable marker therefore was adopted for introduction of unmarked gene deletions in steroid catabolism in *R. erythropolis* SQ1.

As a further embodiment of the present invention, the introduction of a second gene inactivation event can be performed using the same methods as is illustrated in the Examples for *kst*D2. For even further gene inactivation, the same methods may be used again, or, alternatively, UV irradiation or chemical means such as nitroguanidine or diepoxyethaan may be used. Methods to introduce gene mutations in that way are well known in the art.

Also, methods to construct vehicles to be used in the mutagenesis protocol are well known (Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, latest edition). Furthermore, techniques for site directed mutagenesis, ligation of additional sequences, PCR, sequencing of DNA and construction of suitable expression systems are all, by now, well known in the art. Portions or all of the DNA encoding the desired protein can be constructed synthetically using standard solid phase techniques, preferably to include restriction sites for ease of ligation.

Modifications and variations of the method for introducing disrupted gene mutations or unmarked gene deletion as well as transformation and conjugation will be obvious to those skilled in the art from the foregoing detailed description of the invention. Such modifications and variations are intended to come within the scope of present application.

According to another aspect of the present invention micro-organisms possessing multiple gene inactivation's can be used to accumulate steroid intermediates. Preferably the accumulated product is 9α-hydroxy-4-androstene-3,17-dione. The starting material may depend on the enzyme genes which are inactivated. Suitable starting materials are e.g. phytosterols or 4-androstene-3,17-dione. The preferred starting material is 4-androstene-3,17-dione.

An advantage of the present method is that high conversion yields from the starting steroid into the accumulated product can be obtained. The yields may exceed 80%, preferably more than 90% and often reach a value of almost 100%.

Still another aspect of the invention resides in genetically modified micro-organisms with multiple inactivated genes which are involved in steroid degradation. Especially these genes are dehydrogenases. Preferably at least the gene *kst*D1 or *kst*D2 is inactivated. In particular preferred is the inactivation of both genes *kst*D1 and *kst*D2. Preferred are micro-organisms belonging to the genus *Rhodococcus*. Most preferred is the strain *Rhodococcus erythropolis* RG1-UV29.

The micro-organism strains *Rhodococcus erythropolis* RG1-UV29 and *Rhodococcus erythropolis* RG1 have been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany under the accession numbers DSM 13157 and DSM 13156, respectively. These deposits have been made under the terms of the Budapest Treaty.

A person skilled in the art will understand how to use the methods and materials described and referred to in this document in order to construct micro-organisms lacking the ability to degrade the steroid nucleus. Multiple genes encoding for several other steroid nucleus degrading enzymes can similarly be inactivated.

The following examples are illustrative for the invention and should in no way be interpreted as limiting the scope of the invention.

Legends to the figures

Figure 1

Schematic representation of steroid nucleus degradation in *R. erythropolis* SQ1. The positions of the 3-ketosteroid Δ^1-dehydrogenase (KSTD) isoenzymes are indicated with KSTD1 and KSTD3

Figure 2

Schematic representation of the mutagenic vector pSDH422 with the counter-selectable marker *sac*B used for construction of *Rhodococcus erythropolis* strain RG1 with an 1062 bp unmarked *kst*D1 gene deletion. ORF2 and ORF3 are the flanking genes of *kst*D1 in *R. erythropolis* SQ1.

Figure 3

Schematic overview of the molecular organization of *kst*D1 in wild type *R. erythropolis* SQ1 and after integration of pSDH422 by a single cross-over event at the targeted locus downstream (strain SDH422-3) and upstream (strain SDH422-4) of *kst*D1, respectively. Inserted window: Southern analysis, using *kst*D1 as a probe, of *R. erythropolis*

chromosomal DNA digested with *Bam*HI of wild type (lane 1), strain SDH422-3 (lane 2), SDH422-4 (lane 3) and two individual *kst*D1 deletion mutants (lanes 4 and 5).

Figure 4

Bioconversions in 6 liter culture of *Rhodococcus erythropolis* SQ1 UV-29 of 4-androstene-3,17-dione into 9α-hydroxy-4-androstene-3,17-dione. 10 g/l AD (-O-) and 20 g/l AD (-Δ-, -♦-, -X-)

Figure 5

Bioconversion in 6 liter culture of *Rhodococcus erythropolis* RG8 of 4-androstene-3,17-dione into 9α-hydroxy-4-androstene-3,17-dione. 10 g/l AD (-Δ-, -X-)

Examples

Example 1

*kst*D1 characterization.

A degenerated kstD oligonucleotide probe [5' ttcgg(c/g)gg(c/g)ac(c/g)tc(c/g)gc(c/g)tac tc(c/g)gg(c/g)gc(c/g)tc(c/g)atctgg] (SEQ ID NO:2) was developed from an alignment of the N-terminal parts of known KSTD protein sequences of *A. simplex*, *C. testosteroni* and *N. opaca*. Total DNA of *R. erythropolis* SQ1 digested with *Bgl*II was sized by sucrose gradient centrifugation. Southern analysis at 68 °C (stringent washes with 2xSSC for 2x15 min and 0.1xSSC for 2x10 min) of fractions obtained yielded a 6 kb DNA fragment hybridizing with digoxigenine-labelled *kst*D oligonucleotide probe. This fragment was ligated into the *Bgl*II site of the *Rhodococcus-E. coli* shuttle-vector pDA71 (Dabs et al., 1995 Development of improved *Rhodococcus* plasmid vectors and their use in cloning genes of potential commercial and medical importance, p.129-135. In: Proceedings of the Ninth Symposium on the Actinomycetes, Moscow, Russia) and subcloned into *Bam*HI digested pBluescript II KS (Stratagene) (pSDH200).

From restriction mapping analysis we concluded that only one *Eco*RV site was present on the 6 kb fragment, dividing it into equally sized fragments of approximately 3 kb. Southern analysis showed that an approximately 2.9 kb EcoRV fragment of pSDH200 contained sequences homologous to the *kst*D oligonucleotide. Nucleotide sequencing revealed an open reading frame of 1,533 nt (*kst*D1, see SEQ ID NO:1) encoding KSTD1, as was demonstrated by heterologous expression in *Escherichia*. Further

nucleotide sequencing revealed two ORFs of 1,533 nt (ORF1) and 627 nt (ORF2) encoding putative proteins of 510 aa and 208 aa, respectively.

Example 2

KstD1 deletion strain.

A mutagenic vector was constructed that contains a *R. erythropolis* SQ1 chromosomal DNA fragment with a *kst*D1 deletion. A 1062 bp *Bsm*I fragment of pSDH200, encoding a large internal part of KSTD1, was deleted to construct pSDH200)BsmI. For construction of the mutagenic vector a 2724 bp *Sma*I/*Eco*RI fragment of pSDH200)BsmI harbouring the remaining 468 bp of kstD1 and its flanking regions was cloned into the *Sma*I/*Eco*RI site of pK18mobsacB (pSDH422, see Figure 2). The vector pSDH422, encoding kanamycin resistance to select for integration of the mutagenic vector into the chromosome and harbouring the sacB gene of *B. subtilis* for counter-selection, was introduced into *E.coli* S17-1 and mobilized to *R. erythropolis* SQ1 by conjugation as follows. Cells of the *R. erythropolis* SQ1 recipient strain were spread on LBP agar supplemented with 30 $\mu g \cdot ml^{-1}$ nalidixic acid and grown for 5 days. The mutagenic vector pSDH422 was first introduced in *E.coli* S17-1 by transformation.

Transformants (approx. 1000 per plate) grown overnight on selective media (kanamycin 25 $\mu g \cdot ml^{-1}$) were incubated at room temperature for another 24 h. Colonies of both *Rhodococcus* and *E.coli* strains were resuspended in a final volume of 1.5 ml of LBP (1% bacto-pepton (Difco), 0.5% yeast extract (BBL) and 1% NaCl). Aliquots of 750 µl of each strain were mixed and gently pelleted by centrifugation. The pellet was resuspended in 1 ml LBP and cells were spread on non-selective LBP agar in 250 µl aliquots. After growth overnight at 30°C the confluently grown material was resuspended in 2 ml LBP medium and 100 µl aliquots spread on LBP agar supplemented with kanamycin (200 $\mu g \cdot ml^{-1}$) and nalidixic acid (30 $\mu g \cdot ml^{-1}$). *R. erythropolis* SQ1 transconjugants appeared after 3 days. All resulting kanamycin resistant (kan^r) *Rhodococcus* transconjugants were sucrose sensitive (suc^s); no growth occurred after replica plating on LBPS (1% bacto-pepton, 0.5% yeast extract, 1% NaCl, 10% sucrose) agar supplemented with 200 $\mu g \cdot ml^{-1}$ kanamycin.

Southern analysis (Fig. 3) of wild type (lane 1: single band of approx. 4500 bp) and of two transconjugants, SDH422-3 (lane 2: two bands of approx. 2900 and 10100 bp) and SDH422-4 (lane 3: two bands of approx. 4000 and 9000 bp) revealed that both had retained one copy of pSDH422 integrated at the targeted locus by a single recombination event. Gene deletion of *kst*D1 in the *R.erythropolis* SDH422-3 strain was

achieved during growth overnight under non-selective conditions, made visible by subsequent plating on selective medium, i.e. LBPS agar

Gene deletion of the *kst*D1 gene was achieved during growth overnight under non-selective conditions and subsequent plating on selective medium, i.e. LBPS agar. Colony PCR with kstD1 primers (forward primer [5' gcgcatatgcaggactggaccagcgagtgc] (SEQ ID NO:3); reverse primer [5' gcgggatccgcgttacttcgccatgtcctg](SEQ ID NO:4)) on 9 suc^r/kan^s colonies resulted in 6 PCR products with fragment sizes of 468 bp comprising the deleted *kst*D1 gene. Gene deletion was confirmed by Southern analysis at 60 °C (stringent washes with 2xSSC for 2x5 min and 0.1xSSC for 2x5 min) using randomly digoxigenine-labelled *kst*D1 gene as a probe. The 4.5 kb *kst*D1 DNA fragment of wild type obtained after *Bam*HI digestion of chromosomal DNA was reduced to 3.4 kb in the gene deletion mutants, indicating deletion of the expected 1062 bp *kst*D1 DNA fragment. The resulting strain was denoted *R. erythropolis* RG1.

Example 3

Inactivation of steroid Δ^1-dehydrogenation by UV mutagenesis.

Late exponential phase *R. erythropolis* RG1 cells ($2 \cdot 10^8$ CFUs·ml^{-1}) grown in 10 mM glucose mineral medium (K_2HPO_4 4.65 g·l^{-1}, $NaH_2PO_4 \cdot H_2O$ 1.5 g·l^{-1}, NH_4Cl 3 g·l^{-1}, $MgSO_4 \cdot 7H_2O$ 1 g·l^{-1}, Vishniac trace elements, pH 7.2) were sonicated for a short period of time to obtain single cells. Diluted (10^4) samples were spread on glucose mineral agar medium and irradiated for 15-20 sec with an UV lamp (Philips TAW 15W) at a distance of 27 cm, on average resulting in 95% killing of cells. After 4 days of incubation, colonies were replica plated on 4-androstene-3,17-dione (0.5 g·l^{-1}, solubilized in DMSO (50 mg·ml^{-1})) mineral agar medium. Steroid growth deficient mutants scored after 3-4 days were selected for further characterization. To select for strains blocked in Δ^1-dehydrogenation the mutant population was screened for 4-androstene-3,17-dione growth deficient mutants able to grow on 1,4-androstadiene-3,17-dione (0.25 g·l^{-1}) mineral agar medium. It can be concluded that the gene was inactivated. The gene was called *kst*D3 (see Figure 1).

Example 4

Microbiological 9α-hydroxylation of 4-androstene-3,17dione with UV-mutant *Rhodococcus erythropolis* UV-29.

Rhodococcus erythropolis SQ1 UV-29 is a UV-mutant which is capable of conversion of 4-androstene-3,17-dione (AD) into 9α-hydroxy-4-androstene-3,17-dione (9αOH-AD) with concentration of 10 to 20 g/l.

This conversion was performed using the following method:

A 250 ml Erlenmeyer flask containing 75 ml sterile medium (1.5% yeast extract, 1.5% glucose; pH 7.0) was grown with *Rhodococcus erythropolis* SQ1 UV-29 on a rotary shaker (180 rpm) at 28 °C for 24 hours (preculture). A 10 liter fermentor with 6 liter in situ sterilized fermentation broth (1.5% yeast extract, 1.5% glucose, 0.01% antifoaming agent polypropylene glycol; pH 7.5) was inoculated with preculture (1%) and incubated at 28°C for 16 hours under sparging with sterile air and the culture was agitated to induce submerged growth. Then a suspension of 60 gram 4-androstene-3,17-dione in 300 ml polysorbate (0.1%) was introduced. Aerobic incubation with agitation at 28°C was then resumed for 24 hours. Samples of the culture were then extracted with methanol (70%) and filtrated over a dead-end 0.45µm filter before the steroïd composition was determined with HPLC. The same procedure was performed *in triplo* with a two-times higher AD-concentration of 20 g/l, by adding 120 g instead of 60 g AD.

As shown in figure 4 within 24 hours 10-20 g/l of 4-androstene-3,17-dione is almost completely conversed into 9α-hydroxy-4-androstene-3,17-dione (approximately 93% of total 4-androstene-3,17-dione).

Example 5

kstD2 characterization.

A gene library of *R. erythropolis* RG1 was introduced into competent *R. erythropolis* strain RG1-UV29 by electrotransformation. Colonies obtained were replica plated onto mineral agar medium containing 4-androstene-3,17-dione (0.5 g/l) as sole carbon and energy source. Complementation of the strain RG1-UV29 phenotype was scored after three days of incubation at 30 ^{0}C. Colonies growing on 4-androstene-3,17-dione mineral agar medium were cultivated in LBP medium for isolation of plasmid DNA, that was subsequently re-introduced into strain RG1-UV29 to check for genuine complementation. Plasmid pKSD101, isolated from a transformant that showed restored growth on 4-androstene-3,17-dione mineral medium, was introduced into *E. coli* DH5α for further analysis. An insert of approximately 6.5 kb rhodococcal DNA was identified in pKSD101 and subjected to restriction mapping analysis, subcloning and subsequent complementation experiments. A 3.6 kb *Eco*RI DNA fragment of pKSD101 was still able to restore the strain RG1-UV29 phenotype and thus was subcloned in pBluescript(II) KS (pKSD105) for nucleotide sequencing. Nucleotide sequence analysis revealed the presence of a large open reading frame (ORF) of 1,698 nt, encoding a

putative protein of 565 amino acids with a calculated molecular weight of 60.2 kDa. This ORF was designated *kstD2* (SEQ ID NO:5)(which is identical to the previously described *kst*D3 – see Example 3). The deduced amino acid sequence of *kstD2* showed high similarity to known 3-ketosteroid Δ^1-dehydrogenases (KSTD) indicating that *kstD2* encodes a second KSTD enzyme in *R. erythropolis* RG1.

Example 6

kstD2 deletion strains.

R. erythropolis strain RG7 is a mutant strain, obtained from wild type *R. erythropolis* strain SQ1, containing a single *kstD2* gene deletion. *R. erythropolis* strain RG8 is constructed by the successive deletion of two genes encoding 3-ketosteroid Δ^1-dehydrogenase activity, i.e. *kstD1* and *kstD2*, from wild type *R. erythropolis* strain SQ1. Strain RG8 was obtained by deletion of the *kstD2* gene from the genome of the *kstD1* deletion mutant *R. erythropolis* strain RG1. The method used for *kstD2* gene deletion was analogous to the method described for *kstD1* gene deletion in example 2, except for the fact that a different mutagenic vector was used (pKSD201 versus pSDH422).

The mutagenic vector pKSD201 was constructed as follows. A 1,093 bp internal DNA fragment of the *kstD2* gene was deleted by *Mlu*I digestion and subsequent self-ligation of pKSD105, resulting in construction of pKSD200. A 2.4 kb *Eco*RI fragment of pKSD200 harboring the mutated *kstD2* gene was ligated into *Eco*RI digested pK18mobsacB, thereby constructing pKSD201. Plasmid pKSD201 was introduced into *E. coli* S17-1 and mobilized by conjugation to *R. erythropolis* strain SQ1 (to construct strain RG7), or strain RG1 (to construct strain RG8). Transconjugants (suc^s kan^r), resulting from targeted integration of pKSD201 into the genome appeared after 3 days of growth at 30 ^{0}C. Deletion of *kstD2* was achieved by growth of one selected transconjugant (suc^s kan^r) overnight under non-selective conditions (i.e. LBP medium) and subsequent plating on selective LBPS agar medium. Colony PCR performed on 15 suc^r/kan^s colonies with *kstD2* primers (forward primer [5' gcgcatatggctaagaatcaggcaccc](SEQ ID NO:6); reverse primer [5' gcgggatccctacttctctgctgcgtgatg](SEQ ID NO:7)) resulted in 4 PCR products with fragment sizes of 0.6 kb, comprising the remaining part of the *kstD2* gene. Southern

analysis using dig-labeled *kstD2* gene as a probe on *Asp*718 digested chromosomal DNA of wild type and these 4 obtained mutants confirmed deletion of *kstD2*: wild type *Asp*718 DNA fragment of 2.4 kb was reduced to 1.3 kb in the mutant strains.

Example 7

Microbial 9α-hydroxylation of 4-androstene-3,17-dione with *R. erythropolis* strain RG8.

Rhodococcus erythropolis RG8 is a *kstD1* and *kstD2* double deletion mutant which is capable of conversion of 4-androstene-3,17-dione (AD) into 9α-hydroxy-4-androstene-3,17-dione (9αOH-AD) with a concentration of 10 g/l.

This conversion was performed using the following method:

A 250 ml Erlenmeyer flask containing 75 ml sterile medium (1.5% yeast extract, 1.5% glucose; pH 7.0) was grown with *Rhodococcus erythropolis* RG8 on a rotary shaker (180 rpm) at 28 °C for 24 hours (preculture). A 10 liter fermentor with 6 liter in situ sterilized fermentation broth (1.5% yeast extract, 1.5% glucose, 0.01% antifoaming agent polypropylene glycol; pH 7.5) was inoculated with preculture (1%) and incubated at 28°C for 16 hours under sparging with sterile air and the culture was agitated to induce submerged growth. Then a suspension of 60 gram 4-androstene-3,17-dione in 300 ml polysorbate (0.1%) was introduced. Aerobic incubation with agitation at 28°C was then resumed for 24 hours. Samples were taken during the process. These samples were extracted with methanol (70%) and filtrated over a dead-end 0.45μm filter before the steroid composition was determined with HPLC. This process was performed twice.

As shown in figure 5 within 15 hours 10 g/l of 4-androstene-3,17-dione is almost completely converted into 9α-hydroxy-4-androstene-3,17-dione (approximately 92-96% of the total 4-androstene-3,17-dione).

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Akzo Nobel N.V.
Velperweg 76

NL-2824 BM Arnhem

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Akzo Nobel N.V. Velperweg 76 Address: NL-2824 BM Arnhem	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13156 Date of the deposit or the transfer[1]: 1999-11-25

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 1999-11-25 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	Mascheroder Weg 1b D-38124 Braunschweig	U. Weiks Date: 1999-11-29

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 0196

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Akzo Nobel N.V.
Velperweg 76

NL-2824 BM Arnhem

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: RG1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13156

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1999-11-25 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiss Date: 1999-11-29

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 0196

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Akzo Nobel N.V.
Velperweg 76

NL-2824 BM Arnhem

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Akzo Nobel N.V. Velperweg 76 Address: NL-2824 BM Arnhem	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13157 Date of the deposit or the transfer[1]: 1999-11-25

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 1999-11-25 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	Mascheroder Weg 1b D-38124 Braunschweig	U. Weiss Date: 1999-11-29

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 0196

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Akzo Nobel N.V.
Velperweg 76

NL-2824 BM Arnhem

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: RG1-UV29	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13157

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1999-11-25 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

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V. INTERNATIONAL DEPOSITARY AUTHORITY

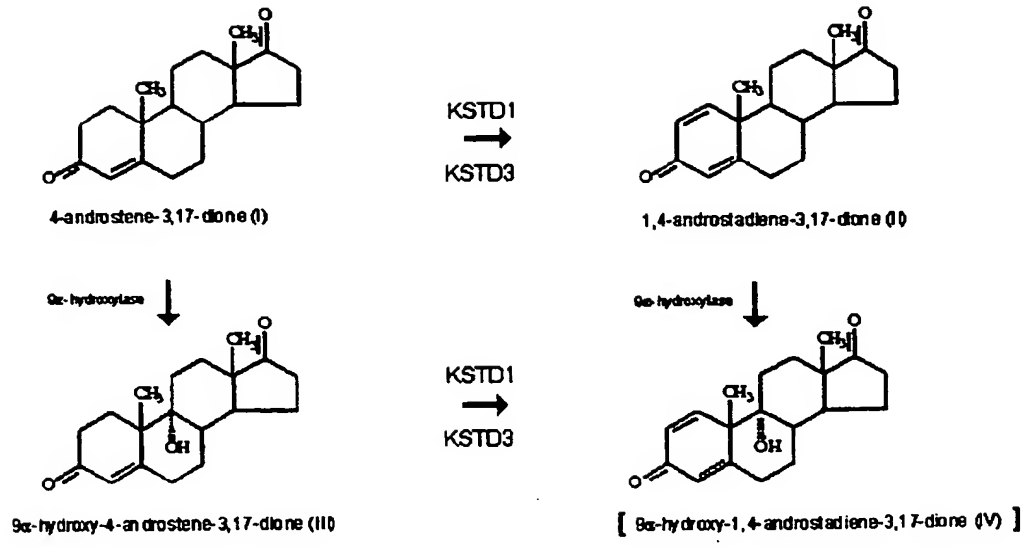
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weiss Date: 1999-11-29

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 0196

Claims

1. Method to construct a genetically modified strain of a steroid-degrading micro-organism lacking the ability to degrade the steroid nucleus, the method comprising inactivation of multiple genes involved in steroid nucleus degradation.
2. Method according to claim 1 wherein the method comprises inactivation of multiple steroid dehydrogenase genes.
3. Method according to claim 2 wherein a first inactivated gene is *kst*D1 or *kst*D2.
4. Method according to claim 3 wherein the first inactivated gene is disrupted or deleted.
5. Method according to claim 3 wherein the first gene is deleted by unmarked gene deletion.
6. Method according to claims 3-5 wherein any subsequent gene is inactivated by UV irradiation.
7. Method according to claims 3-6, wherein any subsequent gene is deleted by unmarked gene deletion.
8. Method according to claim 5, wherein the second gene is deleted by unmarked gene deletion.
9. Method according to claims 1-8 wherein the micro-organism is *Rhodococcus*, preferably *R. erythopolis*.
10. Micro-organism prepared according to claims 1-9.
11. Micro-organism according to claim 10 wherein at least both *kst*D1 and *kst*D2 are inactivated.
12. Genetically modified strain *Rhodococcus erythropolis* RG1-UV29.
13. Use of micro-organisms according to claims 10-12 in the preparation of 9α-hydroxy-4-androstene-3,17-dione by growing said micro-organisms on a culture medium comprising 4-androstene-3,17-dione.
14. Nucleotide sequence encoding KSTD protein encoded by nucleotides 820-2329 of SEQ ID NO:1.
15. Nucleotide sequence encoding KSTD2 protein encoded by nucleotides 1-1678 of SEQ ID NO:5.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KSTD1
KSTD3
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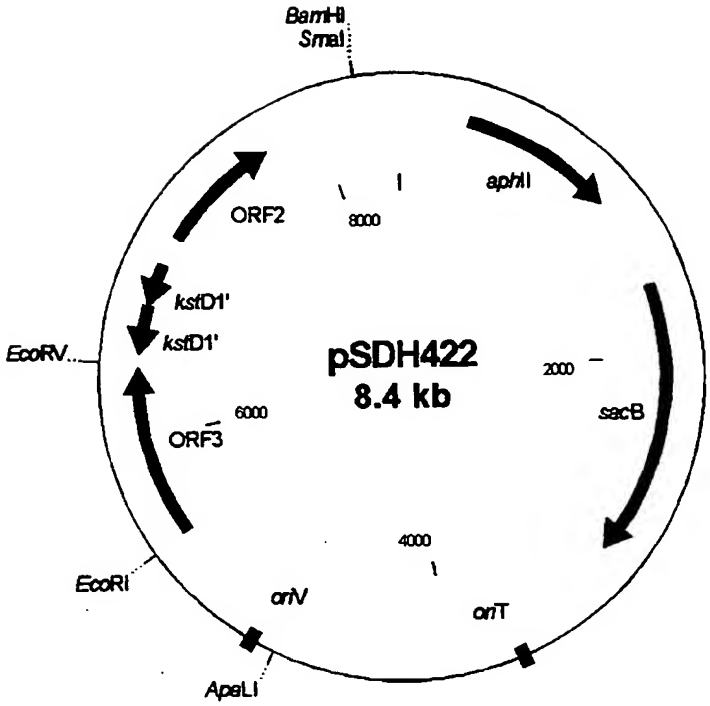
Figure 1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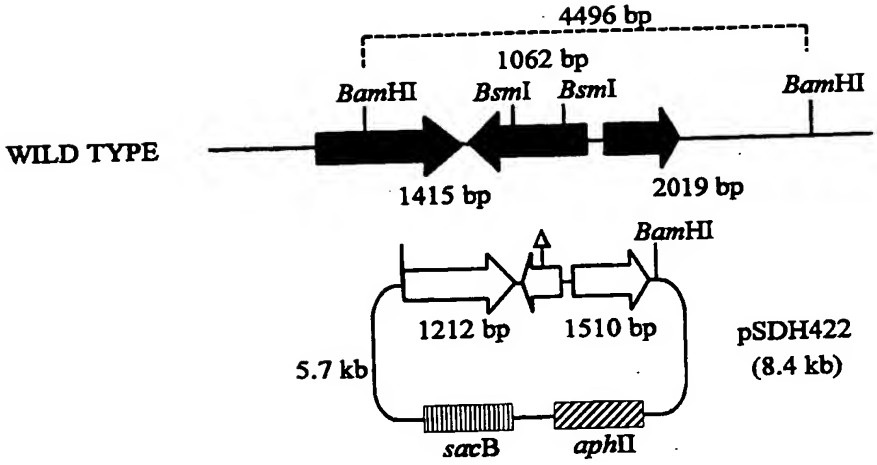
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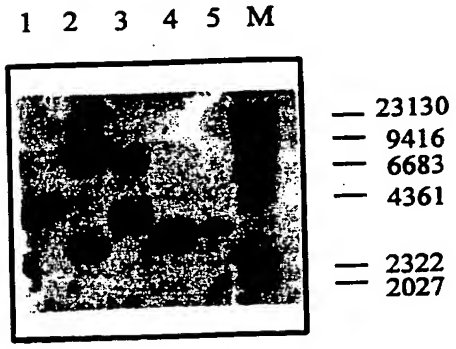
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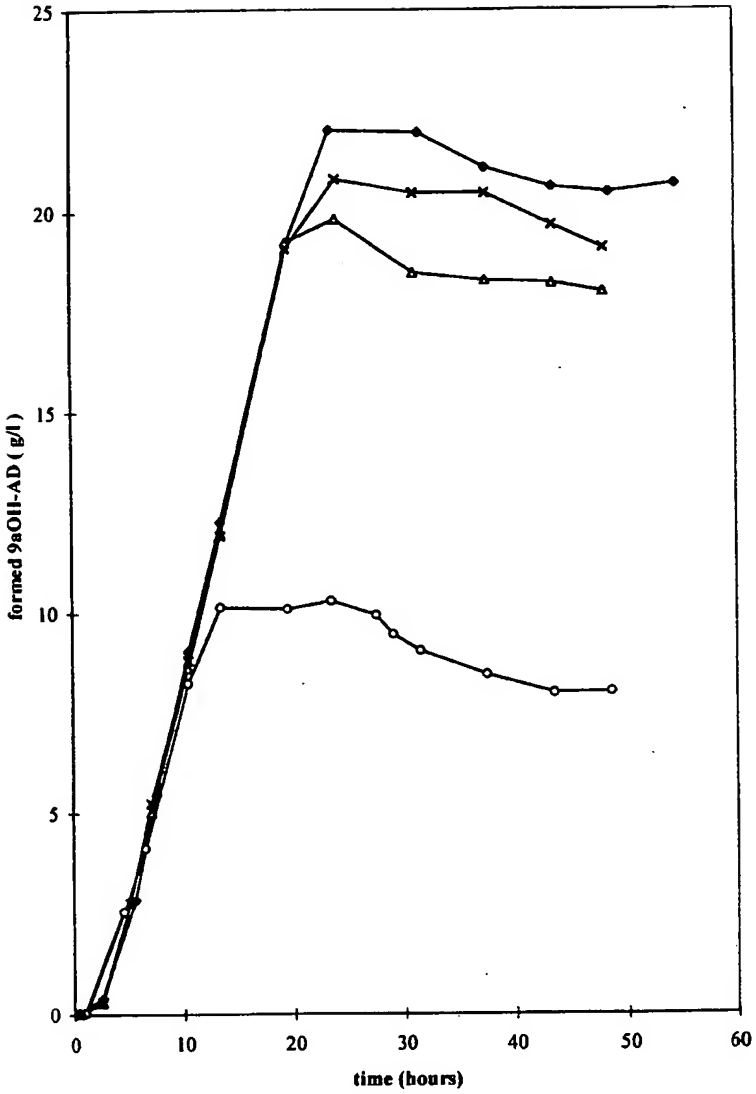

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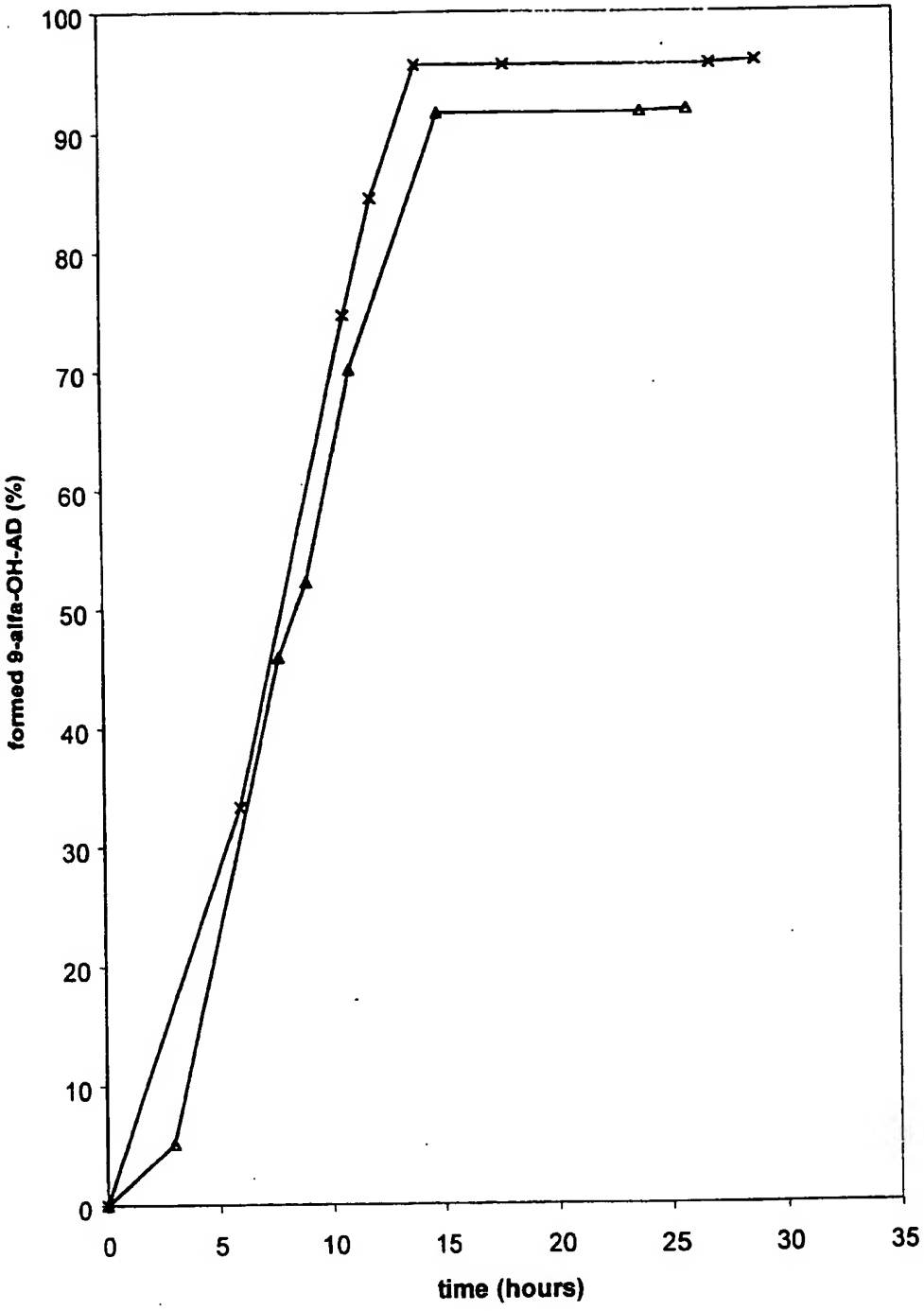
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Figure 5

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INTERNATIONAL SEARCH REPORT

Intern. [redacted]lication No
PCT/EP 00/10223

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P33/00 C12N9/02 C12N15/53 C12N15/63 C12N15/01
C12N1/21 C12N15/76

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 004 695 A (JEKKEL NEE BOKANY ANTONIA ET AL) 2 April 1991 (1991-04-02) column 2, line 1 - line 38 column 5, line 1 - line 36 ---	1,2,10, 13
A	JOHNSTON D M ET AL: "Construction of mutant strains of Neisseria gonorrhoeae lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection" GENE, vol. 236, no. 1, 5 August 1999 (1999-08-05), pages 179-184, XP004175461 ISSN: 0378-1119 page 181, column 1, line 34 -column 2, line 14 --- -/--	5,7,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 2001	29/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Schönwasser, D

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MORII S. ET AL.: "3-Ketosteroid-deltal-dehydrogenase of Rhodococcus rhodochrous:sequencing of the genomic DNA and hyperexpression, purification, and characterization of the recombinant enzyme" JOURNAL OF BIOCHEMISTRY, vol. 124, no. 5, November 1998 (1998-11), pages 1026-1032, XP000891250 the whole document	1-15
P,X	VAN DER GEIZE R. ET AL.: "Targeted Disruption of the kstD Gene Encoding a 3-Ketosteroid deltal-Dehydrogenase Isoenzyme of Rhodococcus erythropolis Strain SQ1." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 66, no. 5, May 2000 (2000-05), pages 2029-2036, XP002157019 the whole document	1-11,13
A	DROBNIC K.: "Steroid 1,2-dehydrogenase; Nocardia opaca 3-oxosteroid delta(1)-dehydrogenase gene, complete cds." EMBL DATABASE ENTRY NO59422; ACCESSION NO. U59422, 25 June 1996 (1996-06-25), XP002157020	14
A	DZIADEK J. ET AL: "Arthrobacter simplex ksdI genes and three open reading frames" EMBL DATABASE ENTRY ASZ93338; ACCESSION NO. Z93338, 25 March 1997 (1997-03-25), XP002157027	15

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1 (incompletely)

Present claim 1 relates to a method defined by reference to a desirable characteristic or property, namely the inactivation of multiple genes involved in steroid nucleus degradation.

The claim covers all methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such methods. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claim also lacks clarity (Article 6 PCT). An attempt is made to define the method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claim which appear to be clear, supported and disclosed, namely those parts relating to methods of inactivating of multiple steroid dehydrogenase genes as mentioned e.g. in claim 2 and on page 1, lines 9 to 33; page 2, line 9 to page 3; line 9; page 5, lines 8 to 10 and examples 2-4, 6 and 7.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

[redacted]ion on patent family members

Interr. [redacted]lication No
PCT/EP 00/10223

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		IT 1218692 B	19-04-1990
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		NL 8702758 A	16-06-1988